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Application of the accelerated test Rancimat to evaluate oxidative stability of dried microencapsulated oils

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RESUMEN

Aplicación del test acelerado Rancimat a la evaluación de la estabilidad oxidativa de aceites microencapsulados

El objetivo de este trabajo es la aplicación del test Rancimat a aceites microencapsulados, con especial interés en el estudio de la eficacia de antioxidantes naturales. Los aceites microencapsulados en matriz seca (DMO) se prepararon mediante liofilización de emulsiones constituidas por caseinato sódico, lactosa y aceite de pescado o girasol, con o sin la mezcla antioxidante ALT (ácido ascórbico, lecitina y tocoferol). En las condiciones seleccionadas en Rancimat (5 g de muestra, 100° C y 20 L/h aire) se obtuvo excelente repetitividad. La mezcla ALT fue mucho más efectiva en el aceite de pescado que en su correspondiente DMO, tanto en Rancimat como a 30°C en la oscuridad. Otros experimentos en Rancimat mostraron que el aumento moderado de la estabilidad en DMO que contenían ALT sólo podía atribuirse al tocoferol mientras que la acción sinergista de la lecitina y el ácido ascórbico no se observó, probablemente porque su efecto depende de su localización y orientación en estos sistemas lipídicos complejos. El test permitió comparar sistemas lipídicos monofásicos (aceites originales y los extraídos de DMO) y heterofásicos (DMO y DMO exentos de la fracción lipídica más accesible) y por tanto ofrece una medida rápida de utilidad para estudiar la influencia de la distribución del aceite y partición de antioxidantes en la estabilidad oxidativa.

PALABRAS-CLAVE: Aceite de girasol - Aceite de pescado - Antioxidante - Estabilidad oxidativa - Microencapsulación - Rancimat - Tocoferol.

SUMMARY

Application of the accelerated test Rancimat to evaluate oxidative stability of dried microencapsulated oils.

The objective of this work was to apply the oxidative test Rancimat to dried microencapsulated oils (DMO), with special emphasis on assessing the efficacy of natural antioxidants. DMO were prepared by freeze-drying emulsions containing sodium caseinate, lactose and fish or sunflower oils, with and without added the antioxidant mixture ALT (ascorbic acid, lecithin and tocopherol). Under the Rancimat working conditions selected for testing DMO (5 g sample, 100°C and 20 L air/h), excellent repeatability was obtained. The antioxidant effect of ALT was much higher in bulk fish oil than in its counterpart DMO, either in Rancimat or at 30°C in the dark. Further experiments using Rancimat showed that the moderate increase in stability of DMO added ALT was only attributable to tocopherol while the synergistic actions of lecithin and ascorbic acid were not observed, their action probably depending on their location and orientation in these complex lipid systems. This test enabled to compare monophasic (bulk oils and DMO-extracted oils) and heterophasic lipidic systems (DMO and DMO devoid of the accessible, free oil fraction), thus offering a rapid means to examine the influence of oil distribution and partitioning of antioxidants on oxidative stability.

KEY-WORDS: Antioxidant - Fish oil - Microencapsulation - Oxidative stability - Rancimat - Sunflower oil - Tocopherol.

1. INTRODUCTION

Dried microencapsulated oils (DMO) are essentially powdery ingredients wherein the oil globules are dispersed in a continuous matrix of saccharides and/or proteins, with the purpose of enhancing stability, controlling release of flavors or masking unpleasant tastes (Dziezak, 1988; Matsuno and Adachi, 1993). In particular, dried microencapsulated fish oils offer important potential applications for dietary supplementation of long chain n-3 polyunsaturated fatty acids (PUFA), of well-known health benefits (Newton, 1996). However, PUFA are highly susceptible to oxidation and, further, other variables pertaining to these heterophasic lipid systems may add complexity in evaluating and controlling oxidative stability. In this context, the need has arisen for analytical techniques useful to study oxidative stability and assess efficacy of antioxidants in DMO (Valenzuela *et al.*, 1993; Haumann, 1997).

Few studies have been reported on oxidative stability of DMO, and these have mainly focused on the influence of microencapsulation agents (proteins, disaccharides, gums, etc.) on oxidation during storage at moderate temperatures (40-60°C), of vegetable oils based-DMO (Fioriti *et al.*, 1975; Sims *et al.*, 1979, Anandaraman and Reineccius, 1986) or fish oils based-DMO (Taguchi *et al*, 1988, 1992; Lin *et al.* 1995). Also, some papers have been published on oxidative changes during long-term storage of PUFA rich-infant formulas at 37°C (Thompkinson and Mathur, 1989, 1990). Recently, we have dedicated our efforts to improve evaluation of oxidation in dried microencapsulated fish oils subjected to storage conditions (Márquez-Ruiz *et al.*, in press). However, one of the aspects in the evaluation of DMO oxidation which may have especial interest but has not been approached so far, deals with the application of rapid, accelerated oxidative tests which enable to predict shelf-life and efficiency of antioxidants.

The accelerated oxidative test Rancimat has been used extensively during the last years to determine oxidative stability of fats and oils under standardized conditions (Rossell, 1994). Concerning lipidic foods, application of Rancimat to intact foods would yield a more realistic representation of what may occur during storage than utilizing the extracted

lipids, with the additional advantage of avoiding any previous, time-consuming handling of samples through the extraction step. Even though little has been published in this regard, some successful applications to intact fried potatoes have been achieved (Barrera-Arellano and Esteves, 1992; Hill, 1994; Jorge *et al.,* 1996; Márquez-Ruiz *et al.,* 1999).

In this work, the Rancimat test has been applied to intact DMO along with bulk oil samples in order to evaluate the efficacy of natural antioxidants, and results compared with those obtained under storage at 30°C.

2. EXPERIMENTAL

2.1. Samples

a) *Oils:*

Fish oil (refined sandeel oil) without added antioxidants and added the mixture of antioxidants ALT, i.e., ascorbic acid (0.03% w/w), lecithin (0.5% w/w) and δ-tocopherol (0.03% w/w) was supplied by the Danish Institute for Fisheries Research (Lyngby, Denmark). Sunflower oil was purchased locally, and utilized «as is» and after elution through an aluminium oxide column (Yoshida *et al.,* 1992) to eliminate tocopherols. Tocopherol free-sunflower oil was added lecithin (0.5% w/w), δ-tocopherol (0.05% w/w) and ascorbic acid (0.03% w/w), while original sunflower oil was added only lecithin and ascorbic acid, following the sequence and procedure of Han *et al.* (1990). Tocopherols were quantitated by normal-phase HPLC with fluorescence detection (IUPAC, 1992) and initial contents of α-tocopherol were 81 mg/kg and 553 mg/kg for fish oil and sunflower oil, respectively. Only trace amounts were found for the other tocopherol isomers.

b) *DMO:*

Microencapsulated oils were prepared starting from a mixture of fish or sunflower oil, D-lactose monohydrate (Sigma, St. Louis, MO, USA) and sodium caseinate from bovine milk (Sigma, St. Louis, MO, USA), each at 10% w/w in deionized water. Emulsions were obtained by homogenization at 10,000 rpm for 5 min. Following freezing at -50°C for 24 h, samples were freeze-dried for 48 h and after milling, samples of powdered dried microencapsulated oils (DMO) were obtained. In DMO samples, the oil phase is distributed into a fraction usually defined as free, surface, accesible or unencapsulated oil, which is easily extractable with organic solvents, and an encapsulated oil fraction which requires disruption of the matrix to be extracted.

c) *DMO devoid of free oil:*

Parallel samples were prepared following extraction of free oil with light petroleum (method described in 2.2.c.). These DMO devoid of free oil contained essentially encapsulated oil.

d) *Oils extracted from DMO:*

Following the procedure described in 2.2.b., total oil was extracted from DMO samples and used in experiments for comparison purposes.

2.2. Characterization of initial DMO samples

a) *Determination of oil globule size:*

Oil globule size distribution was measured in emulsions using a laser diffraction spectrometer (Malvern Mastersizer, Malvern Co., UK).

b) *Extraction of total oil:*

The procedure was based on the Rose-Gottlieb method (Richardson, 1985), widely accepted for quantitative determination of fat in milk and milk powders. Briefly, 1 g of dried ingredient was weighed and dispersed in 10 mL of water at 65°C. After shaking, 2 mL NH_4OH 25% were added and the solution was heated at 65°C for 15 min in a stirring water bath. Then, the solution was cooled, transferred to a separatory funnel and the flask rinsed with 10 mL ethanol. Oil was extracted three times with, first, 25 mL diethyl ether and 25 mL light petroleum, second, 5 mL ethanol, 15 mL diethyl ether and 15 mL light petroleum and third, *idem* without adding ethanol. After filtration through anhydrous Na_2SO_4, solvents were evaporated under reduced pressure and sample dried to constant weight using a stream of nitrogen. Determination of total oil gave relative standard deviations lower than 2% for duplicate analyses.

c) *Extraction of free oil:*

The free oil fraction, also known as accesible, surface or unencapsulated oil, was determined according to Sankarikutty *et al.* (1988). Thus, 8 g of powder were added 200 mL light petroleum (60-80°C) and stirred for 15 min at 25°C in a magnetic stirrer. After filtration through anhydrous Na_2SO_4, solvent was evaporated under reduced pressure and sample dried to constant weight using a stream of nitrogen. Determination of free oil gave relative standard deviations lower than 5% for duplicate analyses.

d) *Calculation of microencapsulation efficiency:*

From the quantitative determinations above detailed, microencapsulation efficiency (g encapsulated oil/100 g total oil) was calculated as follows:

$$\frac{\text{Total oil (g/100 g DMO)} - \text{Free oil (g/100 g DMO)}}{\text{Total oil (g/100 g DMO)}} \times 100$$

2.3. Evaluation of oxidative stability under accelerated conditions

Oil stability index (OSI) was determined at 100°C and 20 mL air/h using a 679 Rancimat apparatus (Metrohm, Herisau, Switzerland) following the AOCS Method (AOCS, 1994).

For intact DMO samples, 5 g (containing approx. between 1.5-1.6 g total oil) were weighed in each reaction vessel, and the reaction vessel attachment was introduced with special care so as to avoid pushing the sample into the narrow glass tube through which air enters. Relative standard deviation for determination of induction period or oil stability index was lower than 3% (n = 3). For DMO devoid of free oil, 5 g were also used (containing approx. 1.1 g oil).

For initial oils and oils extracted from DMO, 1.5 g samples were tested for comparative purposes.

The Rancimat apparatus was used with two evaluation modes, (i) induction period (time corresponding to the inflection point in the oxidation curve) and (ii) time to delta k (tΔk), which is the time needed to achieve a specific difference in conductivity, here selected in 25 μS cm^{-1}.

2.4. Evaluation of oxidative stability under storage conditions

Samples of fish oil based-DMO (2 g) and bulk fish oils (1 g), with and without added ALT, were placed in 10-mL glass vials, sealed and stored at 30°C in the dark. Samples were taken out at selected intervals to monitor evolution of oxidation.

Bulk oils and total oils extracted from DMO were analyzed for polymerization compounds by high-performance size-exclusion chromatography (HPSEC) (IUPAC, 1992b) using a Rheodyne 7725y injector with a 10 μm sample loop, a Waters 510 HPLC pump (Waters Associates, Milford, MA, USA), two 100 and 500 Å Ultrastyragel columns 25 cm x 0.77 cm inner diameter, packed with a porous, highly cross-linked styrenedivinylbenzene copolymer (<10 μm) connected in series, and a refractive index detector (Hewlett Packard, CA, USA). HPLC-grade tetrahydrofuran served as the mobile phase with a flow of 1 mL/min. Sample solutions of 50 mg/mL tetrahydrofuran were used. This analysis enabled rapid quantitation of triglyceride dimers and higher oligomers, here quantitated globally and referred to as polymers.

3. RESULTS AND DISCUSSION

DMO samples gave mean oil globule sizes between 1.42 and 1.57 μm, total oil contents between 31.0% and 32.5% and microencapsulation efficiencies between 70.2 and 72.9%. Microencapsulation efficiency is an important parameter for DMO characterization and depends on a number of process variables, mainly homogenization pressure and solid content of the emulsion, and freezing rate (Flink and Karel, 1970).

Preliminary assays using Rancimat for evaluation of DMO consisted first on assaying different amounts of powders, concluding that 5 g was the maximum amount that guaranteed that sample was entirely surrounded by the heating block and hence temperature was homogeneous. As to selection of temperature, 80°C and 100°C were tested and the latter was found more convenient since evaluations at 80°C were too long and conductivity variations were not sharp enough to allow automatic determination of induction periods by the apparatus. Given that 5 g of DMO and DMO devoid of free oil contained approximately 1.6 g and 1.1 g oil, respectively, the amounts of bulk oils tested in parallel were only of 1.5 g. Once checked that no differences were found between induction periods in the range 1-2 g oil, all samples were accordingly comparable.

Figure 1 shows the typical graphs obtained for samples of bulk oils, DMO and DMO devoid of free oil, in duplicate, corresponding to sunflower oil based-samples. It can be observed that stability increased from the bulk oil to DMO about twice. Also, even at 100°C, DMO devoid of free oil presented a long conductivity curve with gradual increase over time and hence the apparatus did not record the induction period but only time to delta k. In view of these results, it seemed that monitoring of conductivity by Rancimat under the conditions used was primarily sensitive to the most accesible oil fraction.

In order to determine the influence of the amount of free oil in DMO on Rancimat evaluation, several DMO samples were prepared by changing homogenization conditions so as to obtain samples with variable microencapsulation efficiency, from 20.9 to 56.9%, that is, from 1.3 to 0.7 g free oil. No substantial differences were found in induction periods (Figure 2), ranging from 14.1 to 16.2 h, thus supporting the primacy of the accesible, free oil to Rancimat response even at very low amounts, and that responses did not differ in the range of 0.7 to 1.3 g of free oil. Unexpectedly, longer induction period was obtained for sample A (1.3 g free oil) than for similar amount of bulk oil, 1.5 g (Figure 1), which may be related to differences in air accesibility or efficiency of tocopherols in both systems. Such observations were further examined and will be commented later.

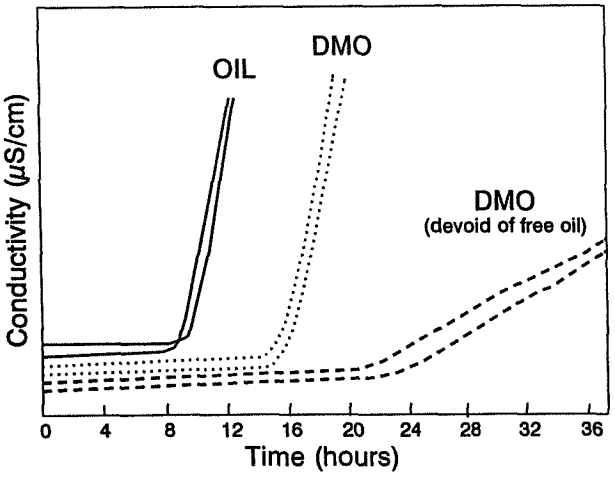

Figure 1
Rancimat plots of sunflower oil-based samples: OIL (bulk oil), DMO (dried microencapsulated oil), and DMO devoid of free oil.

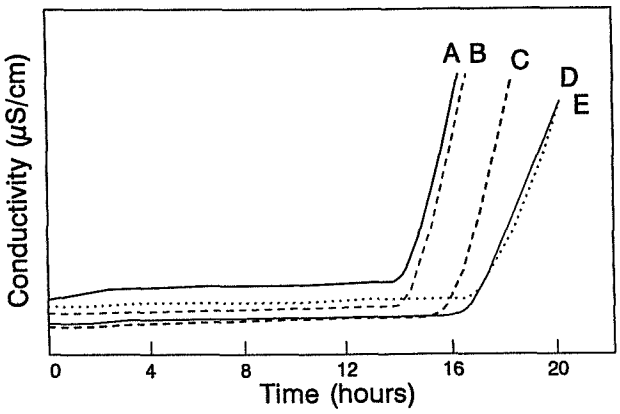

Figure 2
Rancimat plots of dried microencapsulated sunflower oils with different microencapsulation efficiency (ME): A (20.9% ME, 1.3 g free oil), B (56.9% ME, 0.7 g free oil), C (49,0% ME, 0.8 g free oil), D (43.2% ME, 0.9 g free oil) and E (39.1% ME, 1.1 g free oil).

Table I summarizes the results obtained in Rancimat for samples of starting bulk oils, DMO, oils extracted therefrom and DMO devoid of the free oil fraction, here in the case of fish oil-based samples without and with added ALT. ALT was selected as natural antioxidant combination because of its notable efficiency in highly unsaturated oils. Tocopherol and ascorbic acid have been frequently reported as synergistic in their antioxidative properties and combination with lecithin was introduced to help solubilization in bulk oils (Han *et al.,* 1990). The system has proved to extend the induction period of fish oils at 30°C (Han *et al.,* 1991) as well as in Rancimat at 80°C (Yi *et al.,* 1991). Initial fish oils were also tested at 80°C since temperatures lower than 100°C are normally used for fish oils (Kaitaranta, 1992; Méndez *et al.,* 1996). Two evaluations provided by Rancimat (induction period and time to delta k) were included in all samples for comparison with the DMO devoid of free oil, which only gave results on time to delta k. It is important to remark that repeatability was excellent for all types of samples, in both evaluations, although relative standard deviations were always higher for the second evaluation (tΔk). Recently, both Rancimat evaluation modes were compared for assessing relative stability of anchovy, sardine and hake liver oils and results showed that both could be used with the same degree of confidence (Méndez *et al.,* 1997).

In control samples (without added ALT), it was commonly observed that stability increased from the bulk oil to DMO and once the oils were extracted therefrom, similar induction period values than those of the starting oils were obtained. It was clearly observed the significantly enhanced stability of DMO devoid of free oil, thus indicating lower accesibility of air to the encapsulated oil fraction under the conditions used. When ALT was added, stability increased more than 20-fold in the starting fish oil (either at 100°C or 80°C) but decreased considerably once prepared the powder, giving only twice higher protection as compared to DMO without ALT. Likewise, the induction period of extracted oil was only double than that of control and, in consequence, much lower than that of the starting oil. These results support that the antioxidant system ALT was not fully acting in the dried ingredient probably in part due to the poor contribution of the hydrosoluble ascorbic acid. Interestingly, the protective effect of ALT tended

Table I
Rancimat evaluations (h) for fish oil based-samples: induction period (IP) and time to delta k (tΔk)

Sample	Oil		DMO		DMO-extracted oil		DMO devoid of free oil
	IP	tΔk	IP	tΔk	IP	tΔk	tΔk
CONTROL	0.9 (0.1)	1.2 (0.1)	3.9 (0.1)	3.8 (0.2)	0.8 (0.0)	1.1 (0.1)	13.0 (0.3)
ALT	29.1 (0.9)	29.0 (1.1)	13.0 (0.7)	10.6 (1.3)	2.1 (0.1)	2.1 (0.2)	24.4 (0.5)

Values are expressed as Mean (SD) for two determinations.
Abbreviations: DMO, dried microencapsulated oil; CONTROL, without antioxidants and ALT, with ascorbit acid, lecithin and δ-tocopherol.

to be lower in DMO devoid of free oil and extracted oils, both indicative of the encapsulated oil fraction, as compared to DMO, where the free oil fraction was primarily oxidized, as commented above. The relevance of these differences should be carefully examined and further studies are needed to differentiate the effect of ALT in free and encapsulated oil fractions.

Oxidative stability of these highly sensitive fish oil based-samples was also determined under storage conditions. Figure 3 shows the evolution of oxidation for DMO samples with and without the antioxidant mixture ALT at 30°C in the dark. Quantitation of polymers was selected to follow oxidation because it has been recently showed that these compounds are useful markers of the end of the induction period in these highly polyunsaturated oils due to the rapid polymerization occurring at ambient temperature and conditions of high disponibility of air (Márquez-Ruiz *et al.,* in press). Additionally, as compared to polyene index and TBARS, probably the most widely used oxidation measurements of fish oils, evolution of polymers has been found to be more sensitive and consistent (Márquez-Ruiz *et al.,* in press).

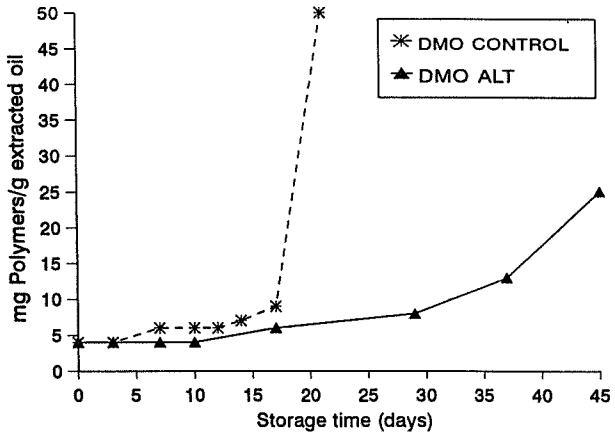

Figure 3
Evolution of polymers in dried microencapsulated fish oils (DMO) without (CONTROL) and with added ascorbic acid, lecithin and δ-tocopherol (ALT), at 30°C in the dark.

The protection provided by ALT under ambient conditions was similar than that obtained in Rancimat, that is, DMO-ALT had twice longer induction time (about 35 days) than control (about 17 days), thus indicating that, under the conditions used, Rancimat was useful to predict the antioxidant efficacy under storage. Even though high temperature-tests have been questioned due to likely changes in the mechanisms of lipid oxidation (Frankel, 1993), some studies have shown high correlations between oxidative stability at room temperature (measured as peroxide development at 20 - 25°C) and stability in Rancimat in a variety of vegetable oils (Gordon and Mursi, 1994; Tekin *et al.,* 1995) or, more related to the present work, between peroxide development in infant formulas at room temperature or 60°C, and stability of the extracted oils in Rancimat (Presa-Owens *et al.,* 1995).

With respect to bulk fish oils, Figure 4 shows the evolution of polymers for the control oil and the oil with ALT. In this case, the end of the induction period was about 40 and 90 days, respectively. Oxidation was considerably more gradual and delayed in bulk oils as compared to DMO samples. Although these observations may seem surprising considering that one of the purposes of microencapsulation is protection against oxidation, it should be noted that a strict comparison between DMO samples and bulk oils is not easy under these conditions because differences in air disponibility between the porous, heterophasic freeze-dried matrix and the monophasic bulk oil may have an important influence. In relation to the efficiency of ALT in bulk oils, it seemed lower at 30°C as compared to the prolonged protection showed in Rancimat. This might be due to the poorer stability of the microemulsion of ALT placed in vials at 30°C in contrast to Rancimat vessels, where te continuous air flow facilitates emulsification.

In order to examine the influence of each component of the ALT antioxidant mixture with more detail, another experiment was conducted using Rancimat, by adding in sequence lecithin, δ-tocopherol and ascorbic acid to sunflower oil devoid of antioxidants, and lecithin and ascorbic acid to the original oil containing naturally occurring α-tocopherol. Results obtained for bulk oils, DMO and DMO-extracted oils are listed in Table II

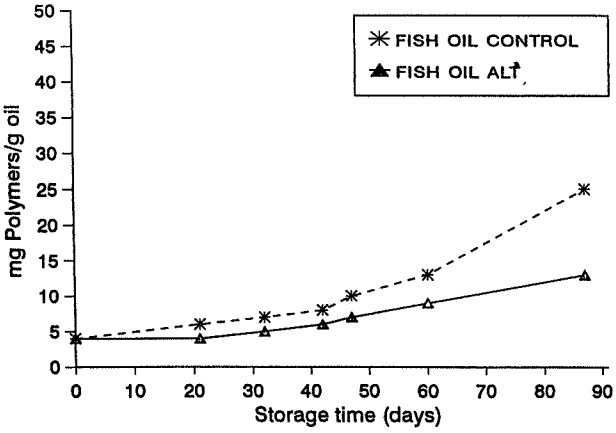

Figure 4
Evolution of polymers in bulk fish oils without (CONTROL) and with added ascorbic acid, lecithin and δ-tocopherol (ALT), at 30°C in the dark.

Table II
Rancimat induction periods (h) for sunflower oil based-samples

	Oil	DMO	DMO-extracted oil
SO (Toc-free)	1.3 (0.0)	1.5 (0.1)	1.1 (0.0)
SO (Toc-free) + LEC	1.3 (0.1)	1.7 (0.1)	1.4 (0.1)
SO (Toc-free) + LEC + δToc	16.2 (0.4)	20.1 (0.9)	6.6 (0.1)
SO (Toc-free) + LEC + δToc	38.2 (1.1)	21.3 (0.8)	7.6 (0.2)
SO	8.3 (0.1)	16.1 (0.7)	7.3 (0.1)
SO + LEC	15.7 (0.5)	20.2 (0.7)	6.1 (0.0)
SO + LEC + AA	35.4 (1.6)	22.0 (1.1)	8.6 (0.1)

Values are expressed as Mean (SD) for two determinations.
Abbreviations: DMO, dried microencapsulated oil; SO (Toc-free), sunflower oil devoid of tocopherol; LEC, lecithin; δToc, δ-Tocopherol; AA, ascorbic acid; SO, original sunflower oil containing α-tocopherol

In the bulk sunflower oil devoid of antioxidants, lecithin alone did not have any antioxidant action, but further addition of δ-tocopherol increased stability about 12-fold and then, an increase in antioxidant effect was observed by adding ascorbic acid. The efficiency of lecithin as synergist of tocopherol was observed for the oil keeping its naturally occurring α-tocopherol, adding here twice as much stability to the bulk oil, and similar induction periods were found for ALT containing-oils, regardless of the different tocopherol isomers. DMO prepared with sunflower oil devoid of tocopherols, both with and without added lecithin, showed similar induction periods than those of their counterpart bulk oils. This observation together with the close results obtained for the DMO samples containing either δ-tocopherol and lecithin or δ-tocopherol, lecithin and ascorbic acid, indicated that the increase in DMO stability was primarily attributable to tocopherol addition. In fact, from the data obtained with original sunflower oil DMO, it was even clearer that only slight increments of DMO stability were due to lecithin or ascorbic acid. It is also important to remark that DMO doubled stability with respect to the original oil (as commented in Figure 1), but only in the case of comparing DMO *vs.* bulk oils containing exclusively tocopherol, indicating that tocopherol was more effective in the accesible, free oil fraction of DMO than in the bulk oil, while further addition of lecithin or ascorbic acid was only efficient in the bulk oil. Finally, in DMO-extracted oils, the increase of stability was virtually provided by tocopherol and, also, it was interesting to observe that the slight increase of induction periods in DMO when lecithin or ascorbic acid were added was here negligible, which might be due to poor extraction.

It has been reported that the effectiveness of antioxidants depends not only on their chemical interactions with compounds of the oxidation pathway but also on their location and orientation in a structured system, thus demonstrating the need for careful consideration of the precise nature of such a system when selecting an appropriate antioxidant combination. Thus, Frankel and coworkers have found that non-polar antioxidants were more effective in oil-in-water emulsions than in bulk oils while the opposite was observed for predominantly polar antioxidants, and these results were attributed to differences in affinities for the air-oil and water-oil interfaces in the two systems (Frankel *et al.*, 1994). In the present study, DMO samples represent lipidic systems alike oil-in-water emulsions but with distinctive characteristics, i.e., although they are prepared from oil-in-water emulsions, other components are added in the formulation and then, water is eliminated to obtain the final dried product.

Among the overall results obtained, it can be concluded that the mixture ALT did not substantially enhance protection in DMO as compared to tocopherol alone. Further studies will be required in order to test the influence of polarity of antioxidants in these complex heterophasic lipid systems, and to differentiate their action in the accesible and encapsulated oil fractions. In this context, the method described here may find an useful application for the rapid assessment of the effectiveness of antioxidants in dried complex products.

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